ABSTRACT

The subcellular distribution of plasminogen activator (PA) was studied in the human fibrosarcoma cell line HT 1080. The cells were homogenized and the cytoplasmic extract (postnuclear supernatant) was fractionated by rate sedimentation and isopyknic equilibration in continuous sucrose density gradients. The distribution of PA was compared with that of a number of subcellular marker enzymes. PA was found to be associated with light, slowly sedimenting particles. It was clearly resolved from the lysosomal marker enzymes β-glucuronidase and N-acetyl-β-glucosaminidase and from catalase used as a marker for peroxisomes. In both fractionation systems, the distribution profile for PA was intermediate between that of the putative plasmalemmal marker enzymes alkaline phosphodiesterase I and leucyl-β-naphthylamidase and that of esterase, a marker of the endoplasmic reticulum. PA-containing structures were resolved to a similar degree from plasmalemma and endoplasmic reticulum fragments by isopyknic equilibration in discontinuous sucrose density gradients. Furthermore, following treatment of the postnuclear supernatant with digitonin, the shift in modal equilibrium density of the PA distribution profile was smaller than that of the plasmalemmal markers and larger than that of esterase.

The consistent dissociation of PA from alkaline phosphodiesterase I and leucyl-β-naphthylamidase suggests that PA is not primarily a constituent of the plasmalemma of transformed cells. It is most likely associated with the membrane of Golgi-derived secretory or carrier vesicles and could this way become incorporated into the plasmalemma and subsequently gain access to the extracellular environment.

The subcellular distribution of PA was also studied in clones of the fibrosarcoma cell line HT 1080 with high or low secretory activity of the cells (13). This suggests that PA secretion in transformed cells does not depend on a particular subcellular distribution of this proteinase and that low secretory activity is not related to increased intracellular storage.

INTRODUCTION

PAs are serine proteases which convert the plasma zymogen plasminogen to its active form plasmin. This enzyme system was initially found to be essential for the lysis of fibrin deposited within the vascular bed (1), and PA was subsequently detected by histochemical techniques in vascular endothelial cells (27). Later, it was discovered that PA was produced by a variety of normal and transformed cells (6). Since plasmin has a broad substrate specificity and plasminogen is present in plasma and interstitial fluids, PAs elaborated by various cells have been implicated in many physiological and pathological processes, e.g., tissue remodeling, cell migration, inflammatory tissue damage, and tumor cell invasion (22).

Recent in vitro studies have demonstrated that the plasmin generated by PAs from tumor cells can degrade extracellular matrix glycoproteins (14) and vascular basement membrane components (16). Among the cells studied were the human fibrosarcoma cell line HT 1080 (21) and clones derived therefrom differing in their rates of PA secretion but having similar levels of cell-associated enzyme activities (15, 16). Both kinds of clones were found to be similarly active against basement membrane preparations, suggesting that degradation was not necessarily dependent on secreted PAs. These observations prompted the present study on the subcellular distribution of PA in the parental cell line and in the clones derived from it.

PA was previously reported to be associated with the plasma membrane of virally transformed fibroblasts (20). More recently, the subcellular localization of PA in transformed 3T3 fibroblasts was found to change in dependence of the secretory activity of the cells (13).

In the present study, PA was recovered in association with small and light subcellular structures which were resolved from plasma membrane fragments in 4 different fractionation systems.

MATERIALS AND METHODS

Cell Cultures

The cell line HT 1080 derived from a human fibrosarcoma (19) was kindly provided by Dr. Rasheed, USC Medical School, Los Angeles. The cells were grown in Eagle’s Minimal Essential Medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum (15) and penicillin and streptomycin (100 IU/ml). Subcultures at a 1:20 dilution were obtained by trypsinization (trypsin:EDTA; Grand Island Biological Co.) of the cells near confluency. Clones secreting high (HT 1080 C+) or low (HT 1080 E−) amounts of PA were obtained from the parental cell line, as described previously (15). The parental cell line and both clones were used for the fractionation experiments.

Fractionation Techniques

Homogenization. Cell monolayers were washed 3 times with a buffered solution containing 140 mM NaCl, 50 mM KCl, 24 mM Tris, and 4 mM Na₂HPO₄, adjusted to pH 7.4 with HCl. They were then scraped from the culture flasks with a rubber spatula into the same buffer and pelleted at 200 × g for 10 min. The cells were resuspended in 15 ml of ice-cold 0.25 or 0.3 M sucrose containing 5 mM HEPES
buffer, pH 7.4, and were disrupted by 6 to 8 strokes in a tight-fitting Dounce homogenizer. A postnuclear supernatant which served as the starting material for subcellular fractionation was then prepared by centrifugation at 1000 × g for 3 min. In all these operations and the fractionations described below, the biological material was kept at 0–6°C.

**Zonal Sedimentation.** A B-XIV rotor (MSE No. 59,144) equipped with a specially designed adapter which reduces the rotor volume from 650 ml to 272 ml (3) was used. The initial conditions in the modified rotor were as follows: 60 ml overlayer consisting of 0.25 M sucrose; 10 ml of sample in 0.3 M sucrose; and a gradient made of six 25-ml portions of 0.4, 0.45, 0.5, 0.55, 0.6, and 0.65 M sucrose, which rested on a cushion of 60% sucrose. All sucrose solutions used contained 5 mM HEPES buffer, pH 7.4. The rotor was filled and emptied at a rate of 20 ml/min while rotating at 2,000 rpm. Sedimentation was performed at either 10,000 or 15,000 rpm for 15 min.

**Isopyknic Equilibration.** A Beaufay rotor (17) which was operated in an MSE SS65 ultracentrifuge (2) was used. Ten ml of postnuclear supernatant, brought to a density of 1.10 by the addition of 60% (w/w) sucrose, were layered onto 30 ml of a linear sucrose gradient extending between the densities 1.17 and 1.29 and resting on a 5-ml sucrose cushion of density 1.32. Centrifugation was carried out at 30,000 rpm for 3 hr. In some experiments, the sample material was treated with digitonin. Digitonin was added to the postnuclear supernate to a final concentration of 0.018% (w/v). After 10 min at 4°C, the density of the preparation was brought to 1.10 and the material was centrifuged under the above conditions. All sucrose solutions used were buffered with 5 mM HEPES buffer, pH 7.4.

**Density Equilibration in Discontinuous Gradients.** Three types of gradients, Gradients A to C, were used. The sample (4.5 ml) consisted of postnuclear supernatants in 33.5, 37, or 40% sucrose. In Gradient A, the sample was overlayed by 4.5 ml of 20% sucrose and rested on three 4-ml layers of 37, 40, and 60% sucrose; in Gradient B, the sample was overlayed as in Gradient A by 4.5 ml of 20% sucrose and rested on three 4-ml layers of 40, 44, and 60% sucrose; in Gradient C, the sample was overlayed by 4.5 ml of 20% sucrose and 4 ml of 37% sucrose and rested on two 4-ml layers of 44 and 60% sucrose. The gradients were made up in 25-ml tubes of a MSE swing-out rotor (No. 59590), and centrifugation was carried out at 29,000 rpm for 16 hr. Fractions were collected from the top of the tubes. All above percentage values are given weight by weight.

**Biochemical Assays**

Protein could not be determined by the method of Lowry et al. (18) because of high blanks due to the presence of HEPES buffer in the samples. In 2 experiments, protein was assayed by the Coomassie blue method of Sedmak and Grossberg (24). PA (23), lactate dehydrogenase (9), and alkaline phosphodiesterase I (10) were measured as described previously. Catatase was assayed at 0°C by the method of Baudhuin et al. (4). Acid 4-nitrophenyl phosphate was determined in 0.05 M sodium acetate buffer, pH 5.3, containing 0.05% Triton X-100 and 5 mM 4-nitrophenyl phosphate. Incubation was carried out for 60 to 90 min at 37°C. The reaction was stopped by addition of 0.1% NaOH, and absorbance was measured at 405 nm (5). N-Acetyl-β-glucosaminidase and β-glucuronidase were assayed in an incubation mixture of 0.2 ml containing 5 mM of the corresponding 4-methylumbelliferyl substrate, 0.05% Triton X-100, and 0.05 mM buffer (sodium citrate, pH 5.0, for N-acetyl-β-glucosaminidase; sodium acetate, pH 4.0, for β-glucuronidase). Following incubation at 37°C for 10 or 30 min, respectively, the reaction was stopped by addition of 3 ml of a 0.05 M glycine:NaOH buffer, pH 10.4, containing 5 mM EDTA. The liberated 4-methylumbelliferone was measured fluorimetrically in a Hitachi-203 fluorimeter (Perkin-Elmer Corp., Instrument Division, Norwalk, Conn.). Leucyl-β-naphthylamide was determined as described by Peters et al. (19) except that the incubation medium consisted of 0.05 M sodium phosphate buffer, pH 7.0, containing 0.0025% Triton X-100. Esterase activity was measured by a modification of the method of Gomori (12), using α-naphthylacetate as substrate. Incubation was carried out in a total volume of 0.7 ml in the presence of 0.05 M sodium phosphate buffer, pH 7.0, 0.008% Triton X-100, and 1 mM α-naphthylacetate (dissolved in ethanol giving a final ethanol concentration of 1.4%). After 15 min at 37°C, the reaction was stopped by adding 1 ml of coupling reagent which was prepared shortly before use by dissolving 100 mg Fast Red TR (Sigma Chemical Co., St. Louis, MO.) in 50 ml of a solution containing 0.2 M acetate buffer, pH 4.5. 10 mM mercury (II) chloride, and 4% Triton X-100. Color was allowed to develop for 15 min at 37°C, and absorbance was measured at 550 nm.

**RESULTS**

**Enzyme Activities.** The specific activities of the marker enzymes in the postnuclear supernatant fraction which was used as the sample for the fractionations are given in Table 1. The homogenization method used resulted in the disruption of more than 50% of the cells. In 5 experiments, PA was determined in the initial cell suspension and the post-nuclear supernatant which was found to contain 53.8 ± 11.3% (S.E.) of this activity. The enzyme activities obtained in corresponding materials from PA-secreting and nonsecreting clones were similar to those of the parent cell line (data not shown).

**Zonal Differential Sedimentation.** Sedimentation experiments were carried out for 15 min at 10,000 and 15,000 rpm. The distribution profiles of PA and other subcellular markers which were obtained in 2 out of 8 experiments of this type are presented in Charts 1 and 2. In both cases, the position of the sample zone is indicated by the distribution of the cytosol marker lactate dehydrogenase, and the approximate boundary between sample zone and gradient is indicated in both graphs by a broken line. At the lower speed (Chart 1), about two-thirds of the activities of 2 putative plasma membrane markers, alkaline phosphodiesterase I and leucyl-β-naphthylamidase, were recovered in the upper half of the gradient in a broad peak partly overlapping the sample zone. The remnant was largely found against the cushion layer. Esterase, a marker for the endoplasmic reticulum (29), was recovered in a sharp, almost symmetrical peak at the boundary between sample zone and gradient. Catalase, a constituent of peroxisomes in many cells.
Chart 1. Fractionation of subcellular components of HT 1080 fibrosarcoma cells by zonal sedimentation at 10,000 rpm for 15 min. Graphs represent normalized distribution histograms as a function of the volume collected. Radial distance increases from left to right. Ordinate, concentration in fractions relative to concentration corresponding to uniform distribution throughout the gradient. Percentage of recoveries were between 92 (PA) and 133 (lactate dehydrogenase).

(7), had an almost identical distribution. As suggested by the position of N-acetyl-β-glucosaminidase, lysosomes sedimented much faster than did the particles mentioned above. At least one-third of the activity had already reached the cushion, about one-half was distributed throughout the gradient, and a small portion, possibly liberated from damaged particles, was retained in the sample zone. β-Glucuronidase, another lysosomal marker enzyme, showed virtually the same distribution as N-acetyl-β-glucosaminidase. The sedimentation profile of PA resembled most closely that of the 2 plasma membrane enzymes. In the upper half of the gradient, however, the shape of the PA profile differed somewhat from that of alkaline phosphodiesterase I and leucyl-β-naphthylamidase. This first experiment already clearly suggested that PA was unlikely to be associated with the endoplasmic reticulum, the particles containing catalase, or the lysosomes.

Sedimentation profiles obtained at higher speed are shown in Chart 2. At 15,000 rpm, the total centrifugal force is slightly more than twice that developed at 10,000 rpm. The 2 plasma membrane markers alkaline phosphodiesterase I and leucyl-β-naphthylamidase had again nearly identical profiles, and the same was true for the 2 lysosomal enzymes N-acetyl-β-glucosaminidase and β-glucuronidase. Most of the activity of the 2 latter enzymes was recovered at the outer limit of the gradient where most of the lysosomes had already accumulated. In contrast to the former experiment (Chart 1), catalase and esterase were well resolved from each other. Catalase had a bimodal profile consisting of a small peak in the starting zone and a major one in the upper third of the gradient, while esterase had a much broader profile extending mainly over the two upper thirds of the gradient and with some activity concentrated at the cushion. As in the low-speed experiments, the distribution profile of PA resembled most closely that of alkaline phosphodiesterase I and leucyl-β-naphthylamidase. Considering the proportion of activity recovered in the gradient and at the cushion, the distribution profile of PA appeared to be intermediate between that of the membrane markers and of esterase.

**Zonal Isopyknic Equilibration.** Average equilibration histograms of the 8 enzymes assayed in the sedimentation experiments are presented in Chart 3. The graphs on the right show the equilibration profiles of the 4 enzymes which were well resolved from PA by zonal sedimentation, lactate dehydrogenase which was retained in the sample zone, catalase which equilibrates in a sharp peak in the upper half of the gradient.
with a small shoulder extending into the sample zone, and the 2 lysosomal glycosidases equilibrating in the middle of the gradient. The distribution profile of PA resembled most closely that of the plasma membrane enzymes and of esterase as was the case in the sedimentation experiments discussed above (Chart 2). It was, however, broader than that of alkaline phosphodiesterase I and leucyl-β-naphthylamidase, and its modal density lay between that of these 2 enzymes and that of esterase, as also shown in Chart 4.

Effect of Digitonin on the Density Distribution of PA and the Membrane Markers. Digitonin was shown to induce an increase in the equilibration densities of membranes in dependence of their content in cholesterol (26). In a further attempt to differentiate the PA-containing particles from other low-density structures, equilibration experiments were performed with postnuclear supernatants of HT 1080 cells which had been exposed to digitonin. The average equilibration profiles obtained and the digitonin-induced equilibrium density shifts are shown in Chart 4. Following digitonin treatment, all profiles were shifted toward higher densities. As expected, since the plasma membrane is particularly rich in cholesterol, the largest shift was that of alkaline phosphodiesterase I. PA shifted to a much lesser extent, and the smallest shift was that of the endoplasmic reticulum marker esterase. A small increase in esterase in the sample zone suggested that to some extent this enzyme had been solubilized by digitonin.

Density Equilibration in Discontinuous Gradients. Since PA and the plasma membrane markers were always close in their distributions, they were compared in a further type of fractionation system. Equal portions of a postnuclear supernatant were adjusted to sucrose concentrations of 33.4, 37.0, or 40.0% (w/w), incorporated into discontinuous gradients of different compositions (see Chart 5) and centrifuged to equilibration. The results are shown in Chart 5. The approximate position of the sample layer is indicated by the peak of lactate dehydrogenase. In all 3 gradients, plasma membrane fragments and lysosomes were clearly resolved; the bulk of N-acetyl-β-glucosaminidase migrated toward the bottom of the gradient while alkaline phosphodiesterase I floated into zones of lower density. PA showed an intermediate behavior. While most of its activity was recovered together with alkaline phosphodiesterase I, there was always some PA activity in clear excess of alkaline phosphodiesterase I in fractions of higher density. These fractionations thus confirmed the trends observed in the density equilibration experiments performed in Beaufays’s rotor.

Subcellular Localization of PA in Secretory and Non-Secretory HT 1080 Clones. The experiments described in the
activity. The similarity, however, was not sufficient to assign PA to the membrane fragments containing alkaline phosphodiesterase I and leucyl-β-naphthylamidase. In fact, its distribution differed slightly but consistently from that of the putative surface markers after sedimentation and isopycnic equilibration in continuous and discontinuous density gradients. Furthermore, the shift in PA modal density induced by digitonin treatment was much smaller than that of the plasma membrane enzymes.

A number of conclusions can be drawn from the effects of digitonin treatment. PA appears to be bound to membranes since it was not released by disruption with the detergent. The membrane fragments containing PA have less cholesterol than do those containing the plasmalemmal markers and more cholesterol than the endoplasmic reticulum, as the equilibrium density shift was intermediate. Furthermore, PA appears to belong to a relatively homogenous population of membrane structures since its equilibration profile was unimodal both before and after digitonin treatment.

Some resolution between PA and a plasma membrane marker, 5'-nucleotidase, was also found by Quigley (20) who concluded that PA is localized in ‘plasma membrane-like elements.’

The dissociation between PA and the plasmalemma markers clearly suggests that PA is not a constituent of the plasma membrane. Its true localization, however, remains speculative. In this respect, interesting suggestions are provided by the results of the digitonin-induced density shift. The extensive subcellular fractionation analysis of the microsomal fraction from rat liver performed by Wibo et al. (30) has shown that membranes derived from the Golgi apparatus undergo a density shift following digitonin treatment which is smaller than that of plasma membrane fragments and larger than that of endoplasmic reticulum and outer mitochondrial membranes. The situation encountered with PA in our experiments is similar, suggesting that PA is localized in Golgi-like membranes. Since PA is produced and secreted by many cells including those which we have investigated (6, 22), it is conceivable that the cell-associated activity is confined to carrier elements, most likely Golgi-derived vesicles. The PA distribution profiles obtained in sedimentation experiments appear compatible with an association of the enzyme with small membrane vesicles which are likely to be slightly heavier and therefore to sediment somewhat faster than plasma membrane fragments (Chart 1). The fact that PA is not liberated in soluble form by the digitonin treatment does not necessarily argue against a localization within carrier vesicles. In fact, PA could be bound at the internal surface of the membrane of the secretory vesicles and become exposed on the cell surface after fusion of the vesicles with the plasmalemma. It could then display its enzymatic action either on the cell surface or after shedding into the pericellular space.

A similar type of intracellular transport has been shown to occur in immunoglobulin-secreting plasma cells (25). An alternative explanation of the resolution between our plasma membrane markers and PA could be that PA is localized in specialized areas of the plasmalemma, with distinct structural and physical properties. However, in view of the well-known lateral diffusion of lipids and proteins within membrane by-layers, such a restriction of PA localization in single cells appears improbable.

Our interpretation of the possible cellular localization of PA
also accounts for the fact that similar subcellular fractionation results were obtained with cells from secreting and nonsecreting clones. In both types of cells, PA appears to be expressed.

REFERENCES

Subcellular Distribution of Plasminogen Activator in Cultured Human Fibrosarcoma Cells

Walter E. Laug, Beatrice Dewald, Joerg Schnyder, et al.